Reference: *Biol. Bull.*, **140**: 461–481. (June, 1971)

HISTOCHEMICAL STUDY OF GUT NUTRIENT RESERVES IN RELATION TO REPRODUCTION AND NUTRITION IN THE SEA STARS, *PISASTER OCHRACEUS* AND *PATIRIA MINIATA*[1]

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Studies of the annual reproductive cycle of the sea stars *Pisaster ochraceus* and *Patiria miniata* (Feder, 1956; Farmanfarmaian, Giese, Boolootian, and Bennett, 1958; Greenfield, 1959; Lawrence, 1965; Mauzey, 1966) have indicated that in *Pisaster* the pyloric caeca show a cyclic variation in size which correlates inversely with size changes in the gonads while in *Patiria* the caeca remain relatively constant in size throughout the gonadal cycle. Biochemical changes in gut lipid, protein, and glycogen in rhythm with the reproductive cycle in these sea stars have been reported in Greenfield, Giese, Farmanfarmaian and Boolootian (1958), Giese (1966a and 1966b) and Allen and Giese (1966).

While biochemical studies give quantitative information about the chemical constitution of various body components, limitations in the method generally make it impossible to locate particular substances within particular tissues or cell types within an organ, and highly localized concentrations of a substance may remain unnoticed (Nimitz and Giese, 1964). Histochemical techniques are capable of localizing chemical components into specific cell types and thus are useful complements to biochemical studies. Histochemical studies are in turn dependent on histological and anatomical studies.

The anatomy and histology of the digestive system of *Patiria* have been described by Anderson (1959, 1960), whose work also includes some histochemical observations. The anatomy and histology of the digestive system of *Pisaster* correspond rather closely to descriptions of the digestive system of *Asterias forbesi* in Anderson (1953, 1954). Mauzey (1966) includes both histological and histochemical observations on the pyloric caeca of *Pisaster*.

The purpose of this paper is to report the results of histochemical investigations on the distribution of carbohydrate and lipid substances in the gut of *Pisaster* and *Patiria* in relation to the reproductive cycle and long term starvation. Observations on the histochemistry of mucins and proteinaceous granules are also reported. Similar studies on the gonads and body wall tissues will be reported elsewhere.

Materials and Methods

Samples of ten sea stars of each species were collected at roughly monthly intervals for two years. The specimens of *Pisaster* were obtained at Duxbury Reef, northwest of San Francisco in Marin County, and the specimens of *Patiria* from White Gulch on the southwest side of Tomales Bay, Marin County. The animals were maintained in laboratory aquaria at approximately 13° C and processed within forty-eight hours.

[1] Supported by USPHS grant GM-12312 to Dominican College.

After each animal was weighed, the gonads and pyloric caeca were weighed in order to obtain the gonad and pyloric caecal indices. The indices are ratios of the weight of the gonads or caeca × 100 to the weight of the animal. The indices of ten animals were averaged to obtain each monthly index except in a

TABLE I

Histological and histochemical techniques employed

Technique	Purpose	Source
Periodic acid-Schiff (PAS)	Most carbohydrates other than acid mucopoly-saccharides	McManus, 1948; Hotchkiss, 1948
Standard sulfation: sulfuric-acetic acid; methylene blue at pH 2.4 (SS)	Carbohydrates other than glycogen	Lewis and Grillo, 1959
Glycogen sulfation: sulfuryl chloride: methylene blue at pH 2.4 (GS)	Glycogen (also stains some other carbohydrates)	Lewis and Grillo, 1959
Lithium-silver nitrate (LiAg)	Glycogen and some mucins	Gomori, 1946; Arzac and Flores, 1949
Alcian blue (AlcBl) and neutral red (NR)	Acid mucopolysaccharides (acidity due to —COOH?)	Steedman, 1950
Azure A metachromasia (AzA)	Acid mucopolysaccharides	Kramer and Windrum, 1954
Methylene blue at pH 2.4 without sulfation (WS)	Sulfated acid mucopoly-saccharides	
Sudan III and IV in acetone-alcohol (SOH)	Neutral lipids	Kay and Whitehead, 1941
Oil red O in isopropanol (ORO)	Neutral lipid	Lillie, 1944
Sudan black B in propylene glycol (SBB)	Neutral lipid	Chiffelle and Putt, 1951
0.1% hematoxylin (CH) at pH 3 and Sudan black B (CSBB) on parallel paraffin sections after chromation	Phospholipids	Elftman, 1954
Sakaguchi's method for arginine (SfA)	Protein (arginine-containing)	Baker, 1947
Coupled tetrazonium reaction using H acid (CT)	Protein	Danielli, 1947
Bromphenol blue (HgBB)	Protein	Mazia, Brewer and Alfert, 1953; Bonhag, 1955
Prenant's triple stain; eosin (E), fast green (FG), and Harris's hematoxylin (HH)	Histology	Gabe and Prenant, 1949
Mallory's phosphotungstic acid-hematoxylin	Histology	

few instances when poor collecting conditions made it impossible to obtain the full quota of animals.

Samples of pyloric caeca, intestinal caeca, cardiac stomach floor and pyloric stomach roof taken from two males and two females each month were fixed in Rossman's fluid for general histology and preservation of carbohydrates and in

TABLE II

Histochemical results. The key to the abbreviations for the staining techniques is in Table I. In addition DL stands for diastase-labile and DR for diastase-resistant

Widely distributed tissues

Peritoneal cell cytoplasm
Fine granules or diffuse staining: FG+; SS+; PAS+; GS+; LiAg+; DL.
Irregular yellow granules: NR+; GS bluish green; AzA-β; CSBB+.
Muscle
Fibers: HH+; NR orange; E+; CH+; CSBB+.
Fiber sheathing
Fine granules: PAS+; GS+; LiAg+; DL.
Fine globules: ORO+; SOH+; SBB+.
Connective tissue fibers: FG+; SS+; PAS+; GS+; AlcBl+; AzA-β.
Mucous cells
Mucus: PAS+; GS+ (often purplish); SS+ (often purplish); AlcBl+; AzA-β; DR.

Cardiac stomach floor

Typical cell cytoplasm
Fine granules or diffuse staining: PAS+; GS+; LiAg+; DL.
Fine globules: ORO+; SOH+; SBB+.
Specialized cell cytoplasm
Fine granules or diffuse staining: PAS+; GS+; LiAg+; DL.
Fine granules at luminal border: GS+ (purple); WS+; AzA-β; CSBB+; SBB brownish black

Pyloric stomach roof

Typical cells
Fine granules or diffuse staining: PAS+; GS+; LiAg+; DL.

Intestinal caeca

Typical cells
Fine granules or diffuse staining: SS+; PAS+; GS+; DL.
Fine globules basally: ORO+; SOH+; SBB+.
Yellow granules, 1–1.5 μ, scattered or in a band: PAS+ rarely.

Pyloric caeca

Storage cell cytoplasm
Basal granules of flagella: E+.
Storage granules; 1 μ, in rows supranuclearly: FG+; CH+; CT+; SfA+.
Fine granules, scattered or in a reticulum or dense reticulum without distinct granules, or diffuse staining: SS+; PAS+; GS+; LiAg+; DL sometimes.
Blotchy deposits: SS+; PAS+; GS+; LiAg grayish brown; DR.
Globules or diffuse staining: ORO+; SOH+; SBB+.
Yellow granules, 0.5–1 μ in a band 4 to 10 μ below border: negative to all stains.
Yellow granules, 0.75–2 μ, in a band 12 to 30 μ below border or scattered supranuclearly: negative to all stains.
"Other granules": E+; AlcBl+; AzA-β.
Zymogen cells
Granules in conical clusters: E+; CH+; HgBB+; CT+.
Typical cells of ducts
Fine granules or diffuse staining: PAS+; GS+; DL.
Fine globules or diffuse staining: ORO+; SOH+.

calcium formol for neutral lipids. Less frequently tissues were fixed in Lillie's neutral formalin for proteins and in 2.5% potassium dichromate in sodium acetate buffer at pH 3.5 for phospholipids. A very few specimens were fixed in Helly's fixative to facilitate study of certain aspects of the histology. Tissues for the study of neutral lipid were embedded in carbowax, other tissues in paraffin, and sectioned at 7 μ.

Table I shows the various staining techniques employed for various purposes and their sources. Control slides for glycogen were incubated in dilute saliva or in 0.01% diastase or amylase in phosphate buffer at pH 6.8 for one to three hours. A few slides of *Pisaster* pyloric caeca were incubated in 0.01% β-galactosidase at pH 7.2, 0.01% β-glucuronidase at pH 7.0, and 0.01% β-glucosidase at pH 5.3 for an hour at 37° C in a series of exploratory tests. A few slides of *Patiria* pyloric caeca were incubated in 0.01% pancreatin at pH 7.0 for one hour.

The samples of tissue from *Pisaster* that had been starved in running sea water tanks at Hopkins Marine Station, Pacific Grove, California, were furnished through the kindness of Dr. A. C. Giese of Stanford University. The specimens of *Patiria* were starved in tanks in the author's laboratory. The water was filtered, aerated and maintained at about 13° C. Sea water and Instant Ocean mix (Aquarium Systems, Inc., Wickliffe, Ohio) were used interchangeably and in combination as convenient.

The starvation for the specimens of *Pisaster* may not have been absolute as some tunicates were discovered living in the aquaria late in the period of starvation (Giese, personal communication). Mauzey (1966) on rare occasions found *Pisaster* feeding on tunicates, though their preferred diet consists of barnacles and molluscs (Feder, 1956; Mauzey, 1966). During starvation both species may have absorbed small amounts of nutrients from natural sea water. Araki (1964) demonstrated the uptake of bovine serum albumin coupled with a diazo salt by *Patiria*, and Ferguson (1967) demonstrated that both *Henricia sanguinolenta* and *Asterias forbesi* absorbed significant amounts of exogenously supplied amino acids and glucose. Even granting the possibilities of nutrients from the sea water or from the stowaway tunicates, it will be apparent that the sea stars under starvation were receiving far less than their normal food intake.

Results

The cardiac stomach of *Pisaster* has five major lobes, each partially subdivided into two portions, one lying on either side of the row of ambulacral ossicles in each arm. The floor of the stomach is thrown into folds radiating from the esophageal region, and these appear in cross section as alternating ridges and gutters.

The wall throughout the digestive tract of *Pisaster* and *Patiria* consists of five layers: a cuboidal epithelium facing the coelom, a layer of muscle fibers, a layer of connective tissue, a nerve layer and the regionally specialized columnar epithelium lining the digestive tract. The basal ends of the columnar cells reach through the nerve layer to attach, often in bundles, to the connective tissue. Amoebocytes with basophilic granules are often seen among the cells of the coelomic lining and in the underlying fibrous layers.

In a study of the cardiac stomach of *Asterias* Anderson (1954) describes "typical cells" with small ovoid nuclei as forming the columnar epithelium of the floor and lower side walls of the gutters. These typical cells are also seen in *Pisaster* in the deeper regions of the gutters. In both *Asterias* (Anderson, 1954) and *Pisaster* specialized cells with elongate nuclei, staining very densely, form the columnar epithelium of the upper side walls of the gutters and intervening ridges, along with mucous cells. As in *Asterias* the line of transition between the typical and specialized cells in *Pisaster* is very abrupt.

At the point near the esophagus where the ridge and gutter system ends and the floor of the stomach becomes a simple, unfolded expanse of tissue in a dissected, relaxed stomach (in the retracted stomach, this region is complexly folded), there is a fairly abrupt transition to a much taller epithelium consisting of very abundant mucous cells intermingled with specialized cells. Present also is another type of secretory cell, reported also by Anderson (1954) in *Asterias*, containing coarse granules which do not stain with phosphotungstic acid hematoxylin. These secretory cells are relatively rare but increase in frequency as one approaches the esophagus proper.

The esophagus in *Pisaster* is most easily distinguished by the fact that its luminal face is regularly ridged, due in part to a folding of the underlying connective tissue and nerve layers and in part to variation in heights of the columnar epithelial cells which are taller in regions overlying the connective tissue folds and shorter between them. The special secretory cells with the refractory granules are relatively abundant in the esophagus and intermingle with mucous cells. However, the most abundant type of cell in the upper region of the esophagus is still the specialized cell with its elongate, densely staining nucleus. Lower in the esophagus the specialized cells are replaced with typical cells containing ovoid granular nuclei. The special secretory cells of the esophagus and adjacent regions of the stomach resemble cells found elsewhere in the external epithelium of the body wall.

This description of the esophagus and cardiac stomach floor in *Pisaster* appears to be in general agreement with Anderson's (1954) descriptions of the corresponding regions of *Asterias*. Anderson, however, describes small granules which stain metachromatically with toluidine blue and are periodic acid-Schiff positive (diastase resistant) at the apical ends of the typical cells (especially those of the lower side walls of the gutters) in *Asterias*. In this present study of *Pisaster* the fine granules staining metachromatically with Azure A appeared to be associated with the specialized cells rather than the typical cells. If present in *Pisaster*, the finely granular cells which Anderson (1954) found in association with the typical cells of the stomach in *Asterias* were not recognized in the present study. Finally, Anderson (1954) does not mention an involvement of the connective tissue layer in the formation of the ridging of the esophagus in *Asterias*.

The columnar epithelium of the roof of the cardiac stomach of *Pisaster* consists of mucous and typical cells, as in *Asterias* (Anderson, 1954).

The pyloric stomach of *Pisaster* is a star-shaped structure with a highly convoluted roof and a simpler floor with five downward folds forming channels radiating from the boundary with the cardiac stomach to the tip of the corresponding lobe. At the tips of the lobes the channels are continuous with short pyloric ducts, each of which soon divides into two and leads into the paired pyloric caeca of each

arm. The pyloric duct is interpreted as beginning where the T-shaped sections of the lobe of the pyloric stomach give way to more oval sections.

A discussion of the histology of the pyloric stomach is most easily begun with consideration of the distribution of cell types in the T-shaped cross sections seen near the junction of each lobe with the pyloric duct. In this region the vertical part of the T is the channel which forms the prominent ridge seen externally when looking at the oral surface of the pyloric stomach.

The epithelium lining the channel consists of typical cells with ovoid, granular nuclei interspersed with mucous cells. The nerve layer is very well developed, especially in the depths of the channel, and the columnar cells tallest on the floor of the channel. The nerve layer thins on the side walls and becomes very inconspicuous at the point where the vertical part of the T meets the cross bar. Mucous and typical cells also form the columnar epithelium of the cross bar, but a third type of cell also occurs here. This is a secretory cell characterized by the presence of very fine basophilic granules and a clear vacuole usually in the basal region. Although these cells in many ways resemble the zymogen cells to be described for the caeca, the granules and vacuoles are much smaller than those of the caecal cells.

As one moves towards the center of the pyloric stomach the roof of the cross bar of the T becomes complexly convoluted. The convolutions result in inverted ridges (reaching down into the stomach) and gutters (folding upwards from the stomach). The cells occupying the floors and side walls of the gutters are very crowded and their granular, ovoid nuclei form a broad band, while the cells on the ridges are apparently less crowded and their ovoid nuclei are confined to a band in the middle third of the epithelium. Mucous cells are fairly abundant in all regions of the convoluted roof. The specialized secretory cells, resembling the caecal zymogen cells but with smaller granules and vacuoles, are also found on the crests of the ridges dipping downward into the lumen. The cells forming the columnar epithelium of the floor of the pyloric stomach are the same as those described above for floor regions nearer the pyloric duct.

In the pyloric duct one finds a continuation of the tracts of the same kinds of cells as are present in the adjacent regions of the pyloric stomach. The lower half of the duct is characterized by tall typical and mucous cells with a well developed nerve layer. In the upper half of the duct the nerve layer is inconspicuous and the typical and mucous cells are accompanied by the specialized secretory (zymogen-like) cells.

The pyloric duct is short and soon divides into two ducts which immediately enter the digestive glands. The main duct of each caecum, once within the caecum, is not to be thought of as a discrete rounded channel but simply as a specialized tract of epithelium along the floor and roof of the organ. Branches of the specialized tracts extend into the large pouches, which in turn may be subdivided into smaller ones. The floor tracts consist of mucous cells and crowded typical cells whose ovoid nuclei form a fairly wide band. Corresponding tracts forming the roof consist of mucous and typical cells accompanied by the specialized secretory (zymogen-like) cells. The nerve and connective tissue layers underlying the duct epithelium are especially well developed, but the columnar cells of the ducts are not as tall as those of adjacent pouch regions.

The histology of the esophagus, cardiac stomach, and pyloric caeca with the accompanying Tiedemann's pouches of *Patiria* have been described by Anderson (1959, 1960). The present paper does not intend to repeat but only to supplement information given there, thus attention will be directed to those aspects of the histology which Anderson (1960) treated most briefly.

The wall of Tiedemann's pouch in each caecum falls into two principal regions, one forming the floor and lower side walls of the pouch, and the other forming the upper side walls of the pouch. The lower region is characterized by a nerve layer which increases in thickness towards the floor of the pouch, by an abundance of mucous cells, and by an abundance of typical cells with finely granular, elongate nuclei which form a band in the middle third of the epithelium. The mucous cells are recognized both because of the pockets containing a secretion staining with fast green in Prenant's triple technique and by similar empty pockets. Above the nerve layer and among the basal portions of the typical cells are seen cells, or portions of cells, containing a densely staining, round nucleus about 2 μ in diameter surrounded by granules about 1 μ across and staining with eosin in Prenant's triple technique. The granules are smaller and more irregular looking when stained with haematoxylin in Mallory's phosphotungstic acid-hematoxylin technique. The first impulse of the present author was to interpret these structures as the lower portions of mucous cells in early stages of secretion for the following reasons: (1) known mucus in the distal pockets stains green in Prenant's technique; (2) granules the same size as the granules which stain with eosin in the basal regions of the epithelium are seen in typical mucous pockets distally, colored either beige (transitional ?) or slightly greenish; (3) it is plausible that the staining reaction of the secretion could change as chemical groups are added or unmasked as the secretory material nears the point of extrusion. On the other hand, these structures containing the eosinophilic granules and small round densely staining nuclei also somewhat resemble the cysts which Anderson (1959) describes in the cardiac stomach of *Patiria,* though Anderson makes no mention of finding such cysts in the Tiedemann's pouches in his 1960 paper. A further consideration is that similar structures with granules (though often not so distinct or as large) and round nuclei are seen in the basal part of the epithelium of the floor of the pyloric duct in *Pisaster,* though not in *Pisaster* stomach. The identity of these structures seems unresolvable with the evidence available.

The upper side walls of the Tiedemann's pouches consist chiefly of very crowded typical cells, the nuclei of which form a band extending over the basal two-thirds of the epithelium. At intervals, as pointed out also by Anderson (1960), groups of mucous cells are seen, corresponding in position to the conspicuous lines running from just above the gutter of Tiedemann's pouch up towards the pyloric caecum externally.

There is very little constriction between the cardiac and pyloric stomachs of *Patiria* and for all practical purposes there is no floor to the pyloric stomach. The roof of the pyloric stomach has ten linear upward folds running towards the entrance of the pyloric duct of each caecum. Minor folds directed outward from the wall of the major folds result in a complexly convoluted roof. The openings into Tiedemann's pouches are continuous above with the openings into the pyloric

ducts and lie just above the circle of fibers which marks the boundary between the cardiac and pyloric stomachs.

The histology of the pyloric duct (consisting of roof and side walls only, since the duct opens into Tiedemann's pouch along its length) resembles that of the roof of the median duct of the caecum. The columnar epithelium contains typical cells which have elongate, granular nuclei occupying a band across the middle third to basal half of the epithelium, and mucous cells. The epithelium of the roof of the pyloric stomach in regions between and on the lower slopes of the upward folds consists of the same two types of cells, but as one moves upwards, toward and into the minor folds, the typical cells become much more crowded and the nuclear band occupies two-thirds or more of the epithelium. The special secretory cells resembling miniature zymogen cells seen in the roof of the pyloric duct of *Pisaster* are conspiculously absent from the duct of *Patiria,* a fact also noted by Anderson (1960).

The histology of the pouches of the pyloric caeca of *Pisaster* and *Patiria* resembles that reported for the pouches of other sea stars (*e.g.,* Anderson, 1953, 1966; Chia, 1969). Mucous cells are found in the epithelium of the pouches of the pyloric caeca, but the predominant cell here is a storage cell with a granular, ovoid nucleus containing a prominent nucleolus. The nuclei form a band a little above the middle third of the epithelium. The storage cells contain numerous granules of different types at different times, and little progress was made in the elucidation of the nature and functions of the different types of granules indicated in Table II, with the exception of the so-called storage granules. These large granules (Fig. 1) are about 1 μ in diameter and occur in a clearly defined row in each cell in the supranuclear cytoplasm. The storage granules were present in most specimens of *Pisaster* except those collected in April and May. Mauzey (1966) found also that the granules were abundant in September but absent in April, and related their presence and absence to "seasonal patterns of transfer and storage of energy-rich materials in the hepatic caeca." (page 139) Chia (1969) found that similar granules in *Leptasterias* disappeared from the pyloric caeca during the brooding period. The storage granules are present in *Patiria* but the seasonal pattern of occurrence is not known.

A third type of cell seen in the epithelium of the pockets of the pyloric caeca is the so-called zymogen cell, reported also by Mauzey (1966) and seen in numerous other sea stars (Anderson, 1953, 1960, 1966; Chia, 1969) though not always with the same distribution as in *Pisaster* (Anderson, 1960). The zymogen cells are most easily identified by their cone-shaped clusters or files of 1.2 μ granules. These clusters are occasionally associated with a small clear vacuole, 2 or 3 μ in diameter, but more often are associated with large clear vacuoles, 8 to 12 μ across, which have presumably enlarged from the smaller size. These large vacuoles were seen in about three-fourths of the specimens of *Pisaster,* sometimes only in a few cells, but sometimes they were so numerous as to form a conspicuous band infranuclearly (Fig. 1). At times when the vacuoles were numerous zymogen granules could not be seen even with the staining techniques which usually show them (Prenant's triple, in which the granules stain with eosin and therefore are presumably acidophilic). In the Azure A technique the vacuoles were occasionally continuous with a narrow basophilic channel which reached apically. Then again the channels, still continuous with vacuoles, could appear empty but with traces of basophilic

material clinging to the edges. Interpreted as possibly a still later stage in a secretory cycle were instances where the narrow channels reaching apically could be seen in cells in which the vacuoles had apparently collapsed. It is therefore proposed that there is a secretory cycle in which the zymogen cells first form the zymogen granules, and that these, in association with the vacuoles, are transformed just prior to release into soluble, enzymatically active fluid, during which transformation the basophilic groups are unmasked. The significance of the vacuole is problematic. The fact that the vacuoles never stained in any test employed whereas both the zymogen granules and secreted fluid did stain (first as acidophilic then basophilic in the present interpretation) seems to render it unlikely that the vacuoles were serving as a site of accumulation of the active material. Also, if there is a growth from small vacuoles to large ones, this could imply that each vacuole is present for a fair period of time. To have active enzymatic material present inside the cell for any length of time seems contradictory to the idea that zymogen granules are a convenient device to avoid intracellular accumulation of enzymes that might digest the cell itself. The second possibility is that after the cell accumulates a supply of zymogen granules, it then accumulates in the vacuole the substance which later acts on the zymogen granules to convert them into active enzymatic material.

Zymogen cells with granules were seen in about 60% of the specimens of *Pisaster* studied during September, October, November, December, March and July, and in 30% or less of the specimens in most other months. This suggests some correlation with the feeding periodicity observed by Mauzey (1966) who also calls attention to the absence of zymogen granules in specimens of *Pisaster* of low pyloric caecal index. Chia (1969) points out that in *Leptasterias* all evidence of secretory activity in the zymogen cells disappears during the brooding season. Starvation for ten weeks does not alter the secretory activity of the zymogen cells in *Leptasterias* (Chia, 1969), and in the present study zymogen granules were observed in about 60% of the starved *Pisaster* and about 33% of the starved *Patiria*.

The attribution of an enzymatic function to the so-called zymogen cells and their granules has rested largely on circumstantial evidence (Anderson, 1966). In the present investigation the granules have been shown to stain with the coupled tetrazonium and the mercury bromphenol blue techniques for proteins, and to the extent that this evidence characterizes the granules as proteinaceous it supports a zymogenic function. (It is recognized that the mercury bromphenol blue procedure must be regarded with reservations (Baker, 1958; Kanwar, 1960).

In *Pisaster* the finger-like intestinal caeca are usually three to five in number, 5 to 15 mm long, and about 2 mm in diameter. In *Patiria* each caecum resembles a cluster of grapes, and the individual lobules are about 1 mm in diameter. In the wall of the caeca the muscle layer is especially well developed. Part of the connective tissue layer is "folded" in such a way that it forms the support for the numerous longitudinal ridges which protrude into the lumen. The columnar epithelium lining the caeca consists chiefly of cells which possess elongate, granular nuclei located in the middle third or lower and often numerous yellowish granules from 0.5 to 2 μ across in the supranuclear cytoplasm. These granules may be scattered irregularly or form a distinct band. Mucous cells are also present. Irregular yellowish granules are sometimes seen in the peritoneal cells.

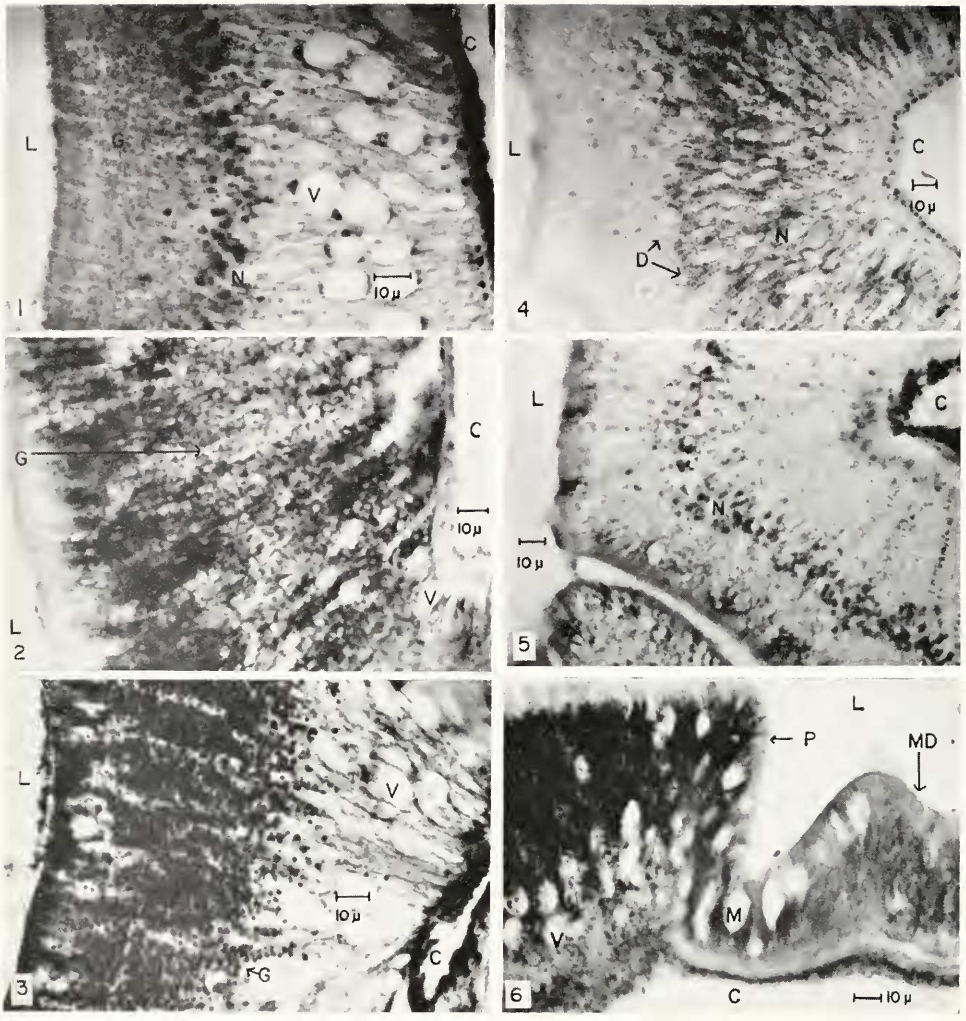

FIGURE 1. Pouch epithelium of the pyloric caeca of *Pisaster*. The nuclei forming a band to the left of the center of the photograph (N) belong to the storage cells, and the granules (G) reaching in files from the nuclear band toward the luminal border (L) are the storage cell granules. The large vacuoles (V) and the nuclei associated with them belong to zymogen cells. The coelom (C) is at the right. This tissue was fixed in Rossman's fluid, embedded in paraffin, sectioned at 7 μ and stained with Prenant's triple technique.

FIGURE 2. Pouch epithelium of the pyloric caeca of *Pisaster*. This tissue was fixed in Rossman's fluid, paraffin-embedded and sectioned at 7 μ, and stained with the periodic acid-Schiff procedure to show the carbohydrate material surrounding the unstained storage granules (G) in the storage cells.

FIGURE 3. Pouch epithelium of the pyloric caeca of *Pisaster*. The storage granules (G) stain deeply with the coupled tetrazonium technique for proteins after fixation in Lillie's neutral formalin, paraffin embedding, and sectioning at 7 μ. Other letters used indicate the same structures as in Figure 1.

From a survey of Table II, summarizing the histochemical staining patterns in the gut of *Patiria* and *Pisaster*, it can be seen that there is evidence for widespread occurrence of a diastase-labile carbohydrate in the tissues of these animals. The carbohydrate material, occurring usually as very fine granules 0.2 μ in diameter, is found in the typical cells of the cardiac stomach floor (and in *Pisaster* also in the specialized cells), in the typical cells of the pyloric stomach roof, basally in the typical cells of the intestinal caeca (though not so consistently here) and in the cells of the duct epithelium of the pyloric caeca. The diastase-labile carbohydrate may well be glycogen, but since current knowledge of the carbohydrates of the invertebrates is still relatively scanty it is perhaps wiser to characterize the carbohydrate that reacts like glycogen as simply glycogen-like. Anderson (1959) also reports a general distribution of glycogen (or a glycogen-like compound) in the stomach of *Patiria;* Anderson (1954) indicates the presence of glycogen in certain regions of the stomach epithelium in *Asterias*.

When very little material reacting positively to stains for carbohydrate is present in the storage cells of the pockets of the pyloric caeca of *Patiria* and *Pisaster,* the carbohydrate here also appears as fine granules labile to diastase, amylase, or saliva. But as the deposits of carbohydrate increase in the storage cells individual granules are no longer recognizable, and the very intensely staining deposits are refractory to diastase, amylase, or saliva to the extent that little or no difference can be seen between slides kept in buffer and slides subjected to one to three hours of enzymatic digestion. This carbohydrate was not labile to β-galactosidase, β-glucuronidase or β-glucosidase in exploratory tests run on *Pisaster* caeca. The intensely staining carbohydrate was absent in sections of *Patiria* caeca after incubation with pancreatin. Anderson (1953) also found that pancreatin removed the diastase-resistant carbohydrate in the caeca of *Asterias* and suggested that the carbohydrate might be bound to protein. Chia (1969) has reported a carbohydrate refractory to diastase in the ground cytoplasm of the storage cells of *Leptasterias*.

The appearance of the carbohydrate material in the storage cells varies in large part due to the presence, absence, or abundance of various kinds of granules and globules refractory to the carbohydrate strains. For instance, at times the carbohydrate may appear simply as fine granules or as a granular reticulum surrounding uncolored storage granules. The reticulum may be so dense that the individual granules composing it are no longer visible (Fig. 2), and there may or may not

FIGURE 4. Pouch epithelium of the pyloric caeca of *Pisaster*. Fine lipid droplets (D) staining with oil red 0 can be seen in the lower two-thirds of the epithelium intermingling with the storage cell nuclei (N). The lumen is at the left, the coelom at the right. The tissue was fixed in calcium-formol; and carbowax sections were cut at 7 μ.

FIGURE 5. Pouch epithelium of the pyloric caeca of *Pisaster*. This tissue was taken from an animal that had been starved for fourteen months, fixed in Rossman's fluid, paraffin-embedded, sectioned at 7 μ, and stained in the periodic acid-Schiff procedure. The nuclei (N) of the storage cells form a prominent band, and there is very little staining for carbohydrate. This figure should be compared to the left half of Figure 6.

FIGURE 6. Pouch (P) and median duct (MD) epithelium of the pyloric caeca of *Pisaster*. This tissue was fixed in Rossman's fluid, paraffin-embedded, sectioned at 7 μ, and stained in the periodic acid-Schiff procedure. Note the intensity of staining in the pouch epithelium; this intensity is typical of the pouch epithelium and is not notably reduced by prolonged diastase- or amylase-incubation. The intensity of staining is much less in the duct epithelium, and the carbohydrate here is diastase- and amylase-labile.

be blotchy deposits of the carbohydrate in the infranuclear region of the cells. At other times the carbohydrate staining of the cells is so uniform as to imply the absence of the refractory storage granules. The amount of carbohydrate may vary from region to region in a single tissue slice, but especially in *Pisaster* it tends to be uniform thoroughout.

This present account of the carbohydrates in the pyloric caeca of *Pisaster* conflicts with Mauzey's (1966) description of carbohydrates in the same species. Mauzey's paper indicates that there are two bands of storage granules supranuclearly in the pocket epithelium and that the more proximal of these bands consists of storage granules which are periodic acid-Schiff positive. Chia (1969) also reports that the storage granules in the storage cells of *Leptasterias* are periodic acid-Schiff positive. The present author characterizes these granules as uncolored by the periodic acid-Schiff technique, though they are embedded in very deeply staining material (Fig. 2). The storage granules were found in the present study to be colored in Sakaguchi's reaction for arginine, the coupled tetrazonium reaction (Fig. 3) and the mercury bromphenol blue reaction, which would suggest that they contain protein. Chia (1969) also reports a protein component in the storage granules of *Leptasterias*.

The more distal band of granules which Mauzey (1966) describes as staining yellow in Mallory's triple technique may be equivalent to the small naturally yellow (*i.e.*, yellow without staining) granules seen in various patterns of distribution in various techniques by the present author.

The present study showed no histochemically detectable variation in the amount of carbohydrate material in the ground cytoplasm of the storage cells of the pyloric caeca, or in any other part of the gut correlating with the reproductive cycle although there is biochemical evidence for such a change (Giese, 1966a and b) which will be discussed in a later section. As mentioned above, Chia (1969) and Mauzey (1966) found that the storage granules, which they characterized as containing carbohydrate, disappeared cyclically, in harmony with the reproductive cycle.

Histochemical results indicate the presence of neutral lipid in the typical and specialized cells of the cardiac stomach floor, typical cells of the pyloric ducts, typical cells of the intestinal caeca, and storage cells of the pyloric caeca (Fig. 4). Anderson (1954) also found lipid in the cardiac stomach epithelium of *Asterias*. In the Tiedemann's pouches of *Patiria* lipid droplets were present in the basal region of the typical cells of the upper side walls, being especially abundant in the cells adjacent to the lines of mucous cells. This is in agreement with Anderson's earlier (1960) observations on the Tiedemann's pouches of *Patiria*. The neutral lipid stains in *Pisaster* and *Patiria* were either taken up by fine globules or appeared to stain diffusely. The deposits were not impressive except in the case of the storage cells of the pyloric caeca. Here discrete droplets of lipid 0.5 to 4 μ in diameter (the larger droplets had probably arisen by the coalescence of smaller ones during processing) occurred almost always in the subnuclear region, and when very abundant, in the supranuclear region as well. Fine lipid droplets were occasionally seen in the peritoneum of the intestinal caeca and stomach.

The distribution of lipids seemed fairly uniform throughout a tissue slice in *Pisaster*, less uniform in *Patiria*. Individual differences in the amount of lipid were observed among specimens in a sample, but no seasonal variation in the amount of lipid was detected histochemically. Chia (1969) found that lipid stores in the pyloric caeca of *Leptasterias* were depleted during brooding, a time during which *Leptasterias* does not feed.

Throughout the gut the connective tissue and the mucins appeared to contain both neutral and weakly acid mucopolysaccharide components, or possibly both

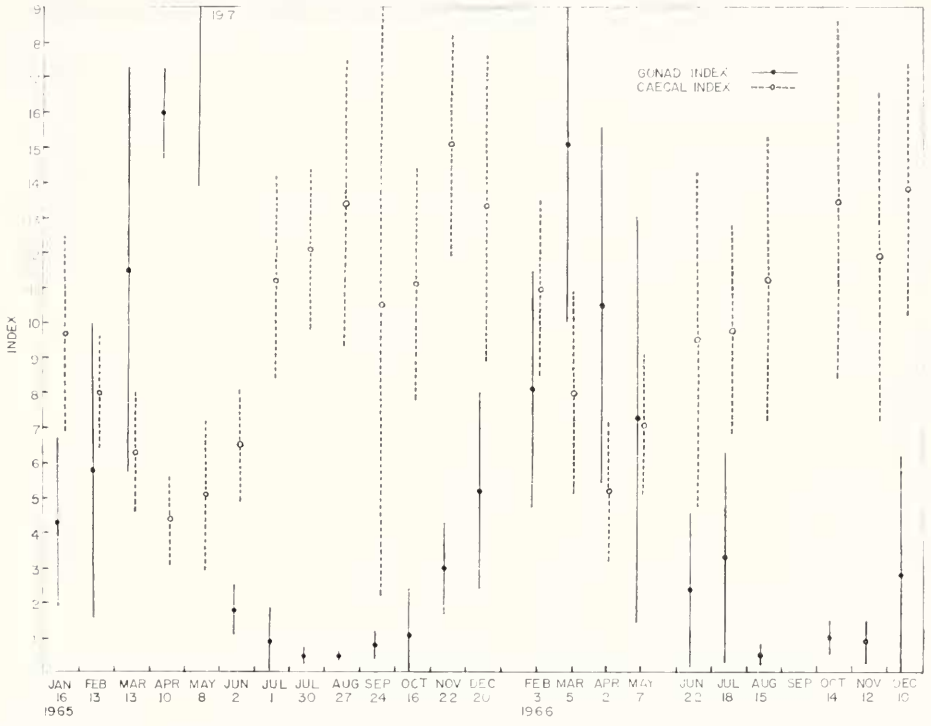

FIGURE 7. Seasonal variation in the gonadal and pyloric caecal indices of *Pisaster ochraceus*. Each mean is indicated by a circle; the standard deviation from each mean is indicated by the vertical line.

neutral and weakly acid residues in a single mucopolysaccharide. Fine lipid globules and granules of glycogen were often associated with muscle fibers of various parts of the gut.

Cyclic changes in the pyloric caeca and changes during starvation

Although no seasonal variations in the amount of neutral lipid and carbohydrate stored in the tissues per unit area were detected histochemically, there is evidence that in *Pisaster* the size (and therefore probably also total amount of stored nutrients) of the pyloric caeca varies during the annual reproductive cycle.

The caeca tend to increase in size from July through December and to decrease in size from January to June (Fig. 7). The reciprocal relationship between the size of the gonads and the size of the pyloric caeca suggests that the increase in size of the gonads during gametogenesis occurs at the expense of materials derived from the caeca, as first proposed by Farmanfarmaian, Giese, Boolootian, and Bennett (1958). Biochemical studies (Giese, 1966a and b) to be discussed later do, however, clearly indicate changes in the lipid and carbohydrate contents of the caeca on a per unit dry weight basis.

The monthly indices for *Patiria* (Fig. 8) show no striking reciprocal relationship between the size of the gonads and caeca. The reason for the lack of

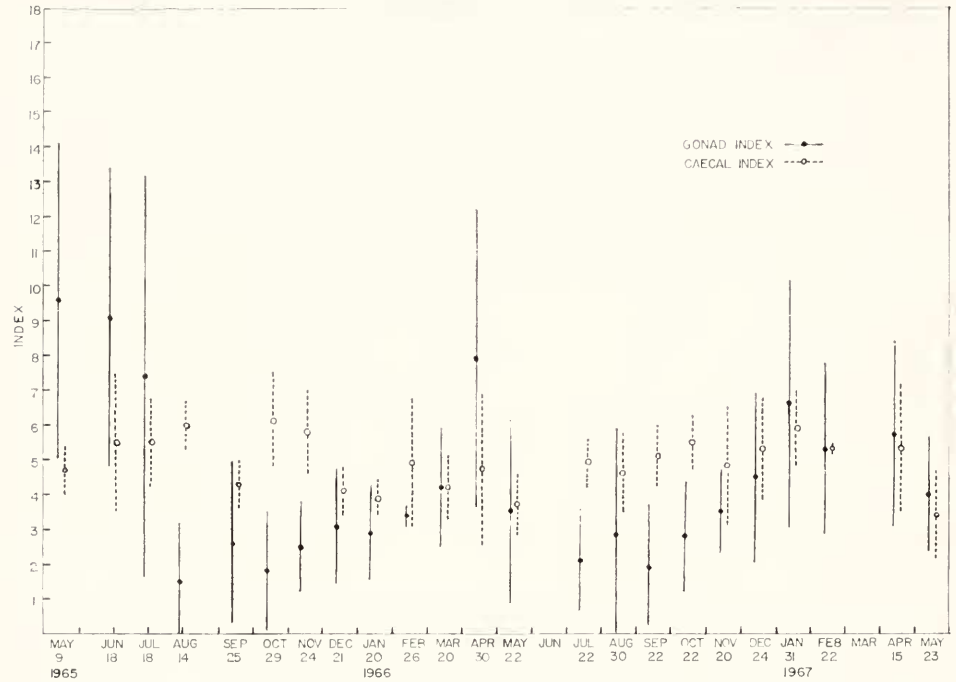

FIGURE 8. Seasonal variation in the gonadal and pyloric caecal indices of *Patiria miniata*. Each mean is indicated by a circle; the standard deviation for each mean is indicated by the vertical line.

reciprocal size relationship is not immediately apparent, but possibly it relates to the omnivorous eating habits of *Patiria*. If suitable food is always available there would seem to be no need to accumulate large stores of nutrients in the caeca prior to the onset of gametogenesis. If periodicity of feeding in *Pisaster* is related to space limitations in the arm and the need to make room for gonadal growth by caecal shrinkage (Mauzey, 1966) the relatively constant size of the caeca in *Patiria* might correlate with more constant feeding. Unfortunately Araki's (1964) study of the feeding habits of *Patiria* did not include a study of the presence or absence of periodicity.

Histochemical studies on animals starved for various periods of time support the role of the pyloric caeca as storage organs.

Six specimens of *Pisaster* were starved from December 15, 1964 to February 7, 1966. Of five stomachs processed successfully, four showed almost normal amounts of diastase-labile carbohydrate, while one contained very little carbohydrate. The neutral lipid content in all was much reduced. Pyloric caeca were processed from six animals. Four animals showed a marked reduction in the amount of storage carbohydrate present (Fig. 5; compare with Fig. 6, left half) one showed about normal amounts of carbohydrate, and one was a mosaic of regions of normal and reduced amounts of carbohydrate. Caecal samples for only five animals were processed for neutral lipids, and all five showed a surprising abundance of neutral lipid. However, instead of being confined to discrete droplets this material was diffusely distributed. Thus a starvation period

TABLE III

Effects of starvation on the gonadal and pyloric caecal indices of Pisaster ochraceus *and* Patiria miniata *(The means are accompanied by the standard deviations.)*

	Wet weight in grams	Gonad index	Pyloric caecum index
Pisaster ochraceus			
10 animals collected and processed January 16, 1965	244 ± 199	4.3 ± 2.4	9.7 ± 2.8
6 animals starved from December 15, 1964 to February 7, 1966	237 ± 50	0.33 ± 0.25	3.6 ± 0.71
6 animals collected and processed February 3, 1966	227 ± 214	5.4 ± 3.3	5.7 ± 1.5
3 animals starved from December 15, 1964 to July 27, 1966	206 ± 44	0.15 ± 0.02	2.2 ± 0.29
10 animals collected and processed July 18, 1966	411 ± 150	3.29 ± 3.02	9.8 ± 2.58
Patiria miniata			
10 animals collected and processed August 30, 1966	157 ± 46	2.8 ± 3.1	4.6 ± 1.2
11 animals starved from August 30, 1966 to May 13, 1967	109 ± 16	1.8 ± 1.3	1.7 ± 0.04
8 animals collected and processed May 23, 1967	148 ± 49	4.0 ± 1.7	3.4 ± 1.3

of fourteen months did reduce the carbohydrate content of the caeca in *Pisaster* but did not appear to reduce the lipid content, although it no longer occurred in discrete droplets after processing.

Three specimens of *Pisaster* were starved an additional six months till July 27, 1966, making a total starvation period of twenty months. Of stomach samples from all three animals, none showed any carbohydrate and only a very few fine droplets of neutral lipid remained. Of the two pyloric caecal samples successfully processed one showed no carbohydrate and the other only small amounts of carbohydrate. Both animals showed a reduced but still goodly amount of neutral lipid present as small globules or coalesced droplets.

About a dozen *Patiria* were starved from August 30, 1966 to May 13, 1967. Of stomach samples taken from four animals three showed about normal amounts of carbohydrate and one very much subnormal amounts. Of three samples processed for lipids, one had subnormal amounts of lipid and two showed no lipid. Of pyloric caecal samples taken from six animals, five showed little or no carbo-

hydrate and one about normal amounts of carbohydrate. Four showed no neutral lipid, one showed a few droplets, and one almost normal amounts of neutral lipid. The starving *Patiria* had become increasingly inactive, thus reducing their metabolic needs to a minimum.

Insofar as quantity can be estimated histochemically, it is apparent that prolonged starvation reduces the amount of carbohydrate and neutral lipid in the storage cells of the pyloric caeca in both *Pisaster* and *Patiria*. The animals apparently draw on carbohydrate stores before mobilizing the lipid reserves, and it is suggested that carbohydrate stores in the stomach are maintained at the expense of stomach lipid, or possibly also at the expense of carbohydrate or lipid in the pyloric caeca. The endurance of *Pisaster* is impressive until one recalls the presence of the tunicates in the tank. Allen (1964) comments on the survival of *Pisaster* during a nine-month fast during which the animals were kept in running sea water from which microscopic algae were not excluded. The caeca lost weight but the percent lipid content of the caeca remained constant, a finding that corroborates the finding of considerable amounts of lipid in the caeca after starvation in the present study. As Allen points out, *Pisaster*, unlike *Patiria*, does not usually evert its stomach in the absence of macroscopic food but the absence of overt signs of microscopical food intake does not preclude its occurrence. Experiments are necessary in which *Pisaster* is deprived of all possible food sources before its survival limits can be accurately measured.

The specimens of *Patiria* sacrificed after nine months of starvation in the present study appeared to be nearing their tolerance limits; in fact, it was because two animals had apparently succumbed to infection, perhaps due to their weakened condition, that the experiment was terminated at nine months. However, Araki (1964) reports the survival of four specimens of *Patiria* for over eighteen months in running sea water without macroscopic food. The animals surely were getting some food in the form of microorganisms, detritus, and dissolved organic matter; however, he found that the intestinal caeca, pyloric caeca, and gonads all looked emaciated.

Anderson (1953) found that eight weeks of starvation depleted the nutrient stores in *Asterias* while Chia (1969) reports an abundance of storage granules, lipid, and ground substance carbohydrate even after ten weeks of starvation in *Leptasterias*.

The storage function of the pyloric caeca in *Pisaster* and *Patiria* becomes even more evident when one reflects on the reduction in size of the caeca during starvation. The magnitude of this reduction can best be appreciated by referring to Table III. It seems reasonable to assume that the pyloric caecal index of the animals placed under starvation initially would have approximated the pyloric caecal index of the animals taken simultaneously from the field and processed at once. Unfortunately, in the case of *Pisaster* no control animals were processed at once, so that the pyloric caecum index for December 15, 1964 is unknown. The best data available are those for a sample of animals collected a month later at a site 150 miles north of the location from which the animals to be starved were taken. The control sample for the effects of starvation on the pyloric caecal index of *Patiria* was collected at the same place and time as the sample of animals to be starved. Comparing the pyloric caecum indices for *Patiria* collected and

processed in August with those starved for nine months, there is a shrinkage from 4.6 ± 1.2 to 1.7 ± 0.04, and for *Pisaster* a shrinkage from 9.7 ± 2.8 in the January "control" animals to 3.6 ± 0.7 after fourteen months of starvation and to 2.2 ± 0.3 after twenty months. In all instances the starved animals had significantly smaller caecal indices than field animals collected at the termination of the starvation experiments. (Again the field animals serving as the controls for *Pisaster* came from a population 150 miles north of where the animals under starvation had originally been collected.)

A second criticism to which the data presented are subject is the difference in average size and range of the starved animals and the field animals to which they are being compared. No work has been done with either species on the relationship of body component indices to body size. Giese (1967) found that in the purple sea urchin, *Strongylocentrotus purpuratus*, some body component indices decreased with increasing size of the animal, while others increased.

Despite these criticisms of the data it still seems reasonable to assert that the digestive glands do shrink during starvation as nutrient reserves are withdrawn for use elsewhere. It may then be asked how this shrinkage takes place. Lawrence, Lawrence, and Holland (1965) have found that in the purple sea urchin starvation results in a decrease of the thickness of the epithelial layer lining all major regions of the digestive tract. They have also hypothesized that fluctuations in the thickness of the columnar epithelium of the gut also account for the annual cycle in the gut index. It seems very reasonable to suppose that changes in the height of the columnar epithelium of the pyloric caeca could account for the annual cycle and starvation shrinkage in sea stars as well, but it is futile to attempt to check on this point with tissues available, since one must work with animals of a limited size range to avoid introducing the additional complication that the height of the epithelium might increase with the size of the sea star.

The increase in size of the gonads in *Pisaster* during the time when the pyloric caeca are shrinking (Fig. 7) is interpreted as suggesting that the material withdrawn from the caeca is made available to the gonads. This interpretation agrees with that of Farmanfarmaian, Giese, Boolootian and Bennett (1958).

Prolonged starvation, which would necessitate utilization of nutrient reserves in support of ordinary metabolic needs as well as those of reproduction, would certainly be expected to have an impact on the spring enlargement of the gonads. The data presented in Table III indicate that the gonads fall far short of their ordinary size increases in starving animals.

Discussion

As mentioned in the introduction, it is particularly instructive if biochemical techniques, which are quantitative, and histochemical techniques, which can localize substances within specific tissues or cells, can be brought to bear on the organic constitution of the same animal. It is the purpose of this discussion to examine the histochemical results of this study in relation to earlier biochemical findings.

Giese's (1966b) data for the stomach show that lipid accounts for 15.4% of the dry weight of the stomach in *Pisaster* and $18.4 \pm 14.4\%$ in *Patiria*. A glycogen-like carbohydrate accounts for 1.5% of the stomach weight in *Pisaster*, 1.1% in *Patiria*. Again the histochemical results are consistent with these data.

There is a consistent staining for glycogen-like carbohydrate (diastase and amylase labile) in the columnar epithelium of the stomach in both species, and an abundance of fine lipid droplets is seen in most specimens. Part of the biochemically detectable lipid would presumably be structural, and relatively invariable in quantity, and the remainder quantitatively variable lipid reserve. Giese (1966b) sets the value of structural lipid in tissues as roughly 5% of the dry weight as a general average.

Giese's data (1966b) for the pyloric caeca show that in *Pisaster* the lipid content varies from roughly 38% in animals of low caecal index to roughly 18% in animals with high caecal index. The caecal indices tend to be lowest in late spring and highest in the late fall, and the fact that the concentration of lipid in the caeca is highest when the caeca are smallest would seem to suggest that when materials are being withdrawn from the caeca for use by the gonads, some other substance contributing to the bulk of the caeca is withdrawn more extensively than lipid. Allen and Giese (1966) found that the rate of incorporation of labeled precursors into caecal lipid rose during October and November and then decreased till June. Again, if the caecal lipid concentration is lowest when the rate of lipid synthesis is highest, it would appear that some other substance is being accumulated in the caeca at a still greater rate. The data in Giese (1966b) suggest that this substance might be protein. Possibly it is both structural protein associated with an increase in height of the columnar epithelium and protein deposited in the storage granules which do disappear cyclically, as reported by Mauzey (1966) and the present author for *Pisaster*. No cyclic variations in lipid content were noted histochemically, but histochemical results indicate that much of the biochemically detectable lipid occurs as neutral lipid droplets in the columnar epithelial cells of the caeca. That this lipid represents nutrient reserve is demonstrated by its decrease in quantity during prolonged starvation in at least some specimens. The pyloric caeca of *Patiria*, which have no pronounced cyclic variation in size are roughly 22% lipid (dry weight).

Giese's (1966a and b) reviews are not concerned with fractionation of lipid, but Allen and Giese (1966) indicate that ordinarily 70% of the hepatic caecal lipid is neutral lipid, and Greenfield (1959) indicates that the maximum nonsaponifiable lipid fraction observed in his study was 50% of the total lipid. This included considerable amounts of a substance with the spectral characteristics of vitamin D, a carotenoid and a sterol fraction showing seasonal fluctuations and capable of influencing gonad development. Karnovsky, Jeffrey, Thomas, and Deane (1955) found that phosphatides amounted to nearly 40% of the total lipid in the caeca of *Asterias forbesi*, with acetal phosphatides and neutral lipids accounting for the remaining fraction. They found abundant neutral lipid droplets by histochemical techniques, but no phospholipid materials were identified histochemically. Karnovsky attributed the failure of Baker's acid hematein test to demonstrate the phospholipids to the low phosphorus content of the phosphatide fraction. The present author was also unable to demonstrate phospholipid granules of any kind in the caeca of *Pisaster* and *Patiria* perhaps for the same reason, though in this case a differential histochemical test was employed.

Histochemical results show a small amount of diastase-labile glycogen-like material in the pyloric caeca accompanied by large deposits of a storage carbohydrate which is not removed by diastase or amylase. As detectedly biochemically,

the content of glycogen-like carbohydrate in *Pisaster* is at maximum 1.7% of the dry weight of the caeca of the male of low caecal index and appears to vary only slightly during the gonadal cycle (Giese, 1966b), while the glycogen-like carbohydrate of the female does show an increase during the cycle from less than 1% in specimens of low caecal index to a maximum of 4.40 ± 3.2% in animals of high caecal index. The content of glycogen-like carbohydrate in the caeca of *Patiria* is also about 1%. The identity of the diastase-refractory carbohydrate is not certain as yet; however, Mr. Arnold Riesen of the author's laboratory has run some preliminary chromatograms of the sugar derived from this carbohydrate. To date, only one sugar appears to be present: glucose. If indeed the carbohydrate proves to be glycogen, it must be assumed that it is bound to some other chemical component in the cells to render it refractory to diastase. As Anderson (1953) has already suggested, this component is possibly protein.

The author wishes to acknowledge gratefully the assistance of the following persons in the processing of the sea stars and preparation of this manuscript: Elizabeth Saghy, Penny Livermore, Charlotte Appel, Charlotte Elmore, Mary Ward, and Sisters Diane Smith, Pamela, Lorraine, and Pietro, O.P. Dr. A. C. Giese and Sister M. Frieda, O.P. have rendered very kind assistance through reading of the manuscript.

Summary

1. Histochemical procedures indicate that in *Pisaster ochraceus* and *Patiria miniata* a diastase-labile carbohydrate occurs in small quantities as fine granules in the peritoneum and muscle of the gut, and in the columnar epithelia of the stomach and ducts of the pyloric caecae. A diastase-resistant carbohydrate occurs abundantly in the storage cells of the pyloric caeca, usually as a matrix surrounding large granules of protein. Neutral lipid droplets occur in small quantities in the columnar epithelium of the cardiac stomach and are abundant in the storage cells of the pyloric caeca.

2. In *Pisaster* the pyloric caeca increase in size from June to December and decrease in size during the spring at the time when the gonads are growing. The inverse size relationship suggests the withdrawal of material from the caeca for use by the gonads. The pyloric caeca of *Patiria* seem to remain fairly constant in size during the breeding cycle.

3. In both species prolonged starvation during the breeding period results in shrinkage of the pyloric caeca and reduction of the nutrient reserves to levels insufficient to support normal gonadal development. Starvation most markedly reduces the histochemically detectable carbohydrate in the pyloric caeca. Stomach lipid is reduced in both species, and most of the caecal lipid disappears in *Patiria*. Even after twenty months of starvation *Pisaster* specimens still showed considerable lipid in the caeca.

4. The connective tissue and mucus from most parts of the gut appeared to contain neutral and weakly acid mucopolysaccharide components or a single compound with both neutral and acid residues. Fine granules found at the apices of cells in certain regions of the stomach contain a more acid mucopolysaccharide.

5. The histochemical results correlate well with earlier biochemical data available for the two species.

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